^{31}P NMR First Spectral Moment Study of the Partial Magnetic Orientation of Phospholipid Membranes

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ABSTRACT Structural data can be obtained on proteins inserted in magnetically oriented phospholipid membranes such as bicelles, which are most often made of a mixture of long and short chain phosphatidylcholine. Possible shapes for these magnetically oriented membranes have been postulated in the literature, such as discoidal structures with a thickness of one bilayer and with the short acyl chain phosphatidylcholine on the edges. In the present paper, a geometrical study of these oriented structures is done to determine the validity of this model. The method used is based on the determination of the first spectral moment of solid-state ^{31}P nuclear magnetic resonance spectra. From this first moment, an order parameter is defined that allows a quantitative analysis of partially oriented spectra. The validity of this method is demonstrated in the present study for oriented samples made of DMPC, DMPC:DHPC, DMPC:DHPC:gramicidin A and adriamycin:cardiolipin.

INTRODUCTION

The spontaneous orientation of phospholipid membranes in magnetic fields has often been considered problematic due to the resulting change in the solid-state nuclear magnetic resonance (NMR) spectral lineshapes (Bayerl and Bloom, 1990; Van Etcheld et al., 1982; Killian and de Kruijff, 1986). However, this phenomenon is now widely exploited since it has been shown to improve the spectral resolution (Sanders and Prestegard, 1990; Vold and Prosser, 1996). Phospholipids spontaneously form unilamellar and multilamellar vesicles, which are usually spherical in an aqueous medium (Helfrich, 1973; Pidgeon et al., 1987), but some membranes, depending on composition, concentration, temperature, and the method of preparation, tend to modify their shape in the presence of a magnetic field (Seelig et al., 1985; Speyer et al., 1987; Jansson et al., 1990; Qiu et al., 1993). When sterols (Brumm et al., 1992; Reinl et al., 1992) or proteins (Neugebauer et al., 1977; Van Echteld et al., 1982; Pott and Dufourc, 1995) are embedded in phospholipid membranes, the orientation may be enhanced or modified.

The origin of the magnetic alignment for a given molecule is its diamagnetic or paramagnetic anisotropy (Boroske and Helfrich, 1978). If a diamagnetic molecule is placed in a magnetic field, a magnetic moment proportional to the diamagnetic anisotropy is induced. Most of the time, the magnetic energy of one molecule is not sufficient to allow its alignment. However, when several molecules are packed together, there may be a reorientation of the aggregate relative to the field (Qiu et al., 1993). The diamagnetic anisotropy of an axially symmetric molecule, $\Delta\chi$, is defined by the difference between the diamagnetic susceptibility parallel ($\chi_{\parallel}$) and perpendicular ($\chi_{\perp}$) to the main axis of the molecule. For molecules with a negative $\Delta\chi$, a perpendicular alignment will occur whereas those with a positive $\Delta\chi$ will orient parallel to the field. For example, in phospholipids, the hydrocarbon chains, which are considered to be the reference axis, have a $\Delta\chi < 0$ (Boroske and Helfrich, 1978), whereas glycerol ester carbonyls have a $\Delta\chi > 0$ (Maret and Dransfeld, 1985). Because glycerol carbonyls are about perpendicular to the phospholipid hydrocarbon chains (Sakurai et al., 1980), they both tend to orient the long axis of the lipid at 90° relative to the magnetic field. Peptide bonds also have a positive $\Delta\chi$ (Worcester, 1978). Because all peptide bonds contained in α or β helices are parallel to the helix axis, helical proteins will tend to orient parallel to a magnetic field (Neugebauer et al., 1977).

Proteins can also contain aromatic residues that are known to have large negative $\Delta\chi$ (Maret and Dransfeld, 1985). In particular, gramicidin A (GA) has 4 tryptophan residues in which the planes of the aromatic rings are approximately parallel to the helix axis (Ketchem et al., 1997), favoring a parallel orientation. In the same way, an aromatic molecule such as adriamycin (ADM) is expected, due to its negative $\Delta\chi$, to orient its plane parallel relative to a magnetic field. In fact, it has been shown that the anthraquinone moieties of ADM molecules are closely stacked together when they are present at high concentration on the surface of a negatively charged phospholipid membrane (Goormaghtigh and Ruysschaert, 1984; De Wolf et al., 1991). Their magnetic susceptibilities are therefore added up, which makes possible the orientation of the membrane. The orientation of this membrane will depend on the angle formed by the ADM and the normal to the bilayer. If the ADM molecule is tilted by 39° (Goormaghtigh et al., 1987), the membrane would orient its normal parallel to the magnetic field.

The shape of phospholipidic membranes under high magnetic field is usually prolate ellipsoid (Helfrich, 1973; Speyer et al., 1987). These structures have been observed by electron microscopy (Brumm et al., 1992), ^{31}P NMR (Pott and Dufourc, 1995; Brumm et al., 1992) and ^{2}H NMR

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spectroscopies (Reinl et al., 1992; Schaefer et al., 1998). In addition, specific phospholipid mixtures are known to form bicelles, which are discoidal micelles formed when a long chain lipid (such as dimyristoylphosphatidylcholine (DMPC)) is mixed either with a short chain lipid (such as dihexanoylphosphatidylcholine (DHPC)) (Sanders and Schwonek, 1992; Vold and Prosser, 1996; Sanders and Prosser, 1998), a bile salt such as sodium glycocholate (Ram and Prestegard, 1988), or the detergent 3-(cholamidopropyl)dimethylammonio-2-hydroxy-1-propanesulfonate (CHAPSO) (Sanders and Prestegard, 1990; Hare et al., 1995). In addition, it was recently observed that the addition of small amounts of paramagnetic ions to pure bicelles results in systems in which the director is oriented parallel to the magnetic field (Prosser et al., 1996). This phenomenon is due to paramagnetism and will not be considered here.

Solid-state ^{31}P NMR spectroscopy is an ideal technique to follow the orientation of phospholipid membranes due to the presence of only one phosphorus atom in each phospholipid and the absence of phosphorus in most proteins. In addition, several interactions studied by ^{31}P NMR are orientation dependent, such as the dipolar coupling and the chemical shift anisotropy (CSA) (Seelig, 1978; Smith and Ekiel, 1984). Spectral simulations have been done to extract the ratio of ellipsoid long/short axis from experimental ^{31}P (Pott and Dufourc, 1995; Brumm et al., 1992) and ^{2}H NMR spectra (Reinl et al., 1992; Schaefer et al., 1998). In contrast, even though ^{31}P and ^{2}H NMR spectroscopies have been applied to bicelles, no spectral simulation of such system has been done to our knowledge. However, the shape of the bicelles has been investigated by Chung and Prestegard (1993) based on field gradient studies and by Vold and Prosser (1996) based on the ratios of the deuteron splittings for DHPC and DMPC.

In the present paper, a method is proposed that uses the first spectral moment of ^{31}P NMR spectra to obtain structural details on magnetically oriented phospholipid membranes. More specifically, it can be used to determine either the dimension of a bicelle or the ratio of an ellipsoid long/short axis. All these parameters are extracted from equations that are derived in the Theory section. Different systems are studied to present several of the possibilities mentioned above. First, DMPC forms ellipsoidal vesicles in which the phospholipids have a perpendicular orientation relative to the magnetic field. Moreover, a DMPC:DHPC mixture gives bicelles that align the bilayer normal perpendicular to the magnetic field. Finally, the lipids in membranes made of DMPC:DHPC and GA have a parallel orientation relative to the field, such as those in a complex made of a mixture of cardiolipin (CL) and ADM.

THEORY

In axially symmetric systems, ^{31}P NMR chemical shifts are defined as

$$\omega = \delta \frac{(3\cos^2\beta - 1)}{2} + \delta_{iso}, \quad (1)$$

where β is the angle between the principal axis of the chemical shift tensor and the static magnetic field, and δ_{iso} is the isotropic chemical shift. To simplify this equation, the CSA parameter, δ, and the isotropic chemical shift are expressed in frequency units. Due to this chemical shift orientation dependence, both the spectral lineshape of a static sample and the weighted-average frequency of a sample with fast molecular motions can be related to the orientation distribution of the molecules in the sample. Therefore, if partial orientation occurs during an NMR experiment, a modification of either the lineshape or of the weighted-average frequency will also be present. Thus, information about the orientation distribution can be obtained from a detailed analysis of the spectral modification.

For static samples, a relationship between the spectral density, $S(\omega)$, and the angular distribution, $P(\beta)$, called the principle of differential conservation of the integral (Schmidt-Rohr and Spiess, 1994) is given by

$$S(\omega)|d\omega| = P(\beta)|d\beta|. \quad (2)$$

That implies

$$S(\omega) = \frac{P(\beta)}{|d\omega/d\beta|} = \frac{P(\beta)}{|3\delta\cos\beta\sin\beta|}. \quad (3)$$

In a randomly distributed sample, the angular distribution is easily calculated and the weighted-average frequency is equaled to the isotropic chemical shift, but, in lyotropic liquid crystals, partial orientation can occur due to the diamagnetic anisotropy. In this case, the orientational distribution has to be calculated. In the next section, two cases of partial orientation will be considered, an ellipsoid orientation distribution and a discoidal orientation distribution called bicelles.

Angular distribution and spectral lineshape

The angular distribution for ellipsoids has already been investigated by Pott and Dufourc (1995). Our approach is very similar to theirs but the following presentation is still necessary for a better understanding of that used for bicelles. Also, the description of the origin of the ellipsoidal angular distribution will help to define the effect of partial orientation on the first spectral moment.

Ellipsoids

Ellipsoids can be represented by the parametric equations,

$$x = a\cos\theta\sin\varphi,$$

$$y = a\sin\theta\sin\varphi,$$

$$z = c\cos\varphi, \quad (4)$$

where the semiaxes a and c and the angles φ and θ are represented in Fig. 1. The ellipsoid is called either a prolate spheroid if the semiaxis c is greater than the semiaxis a or

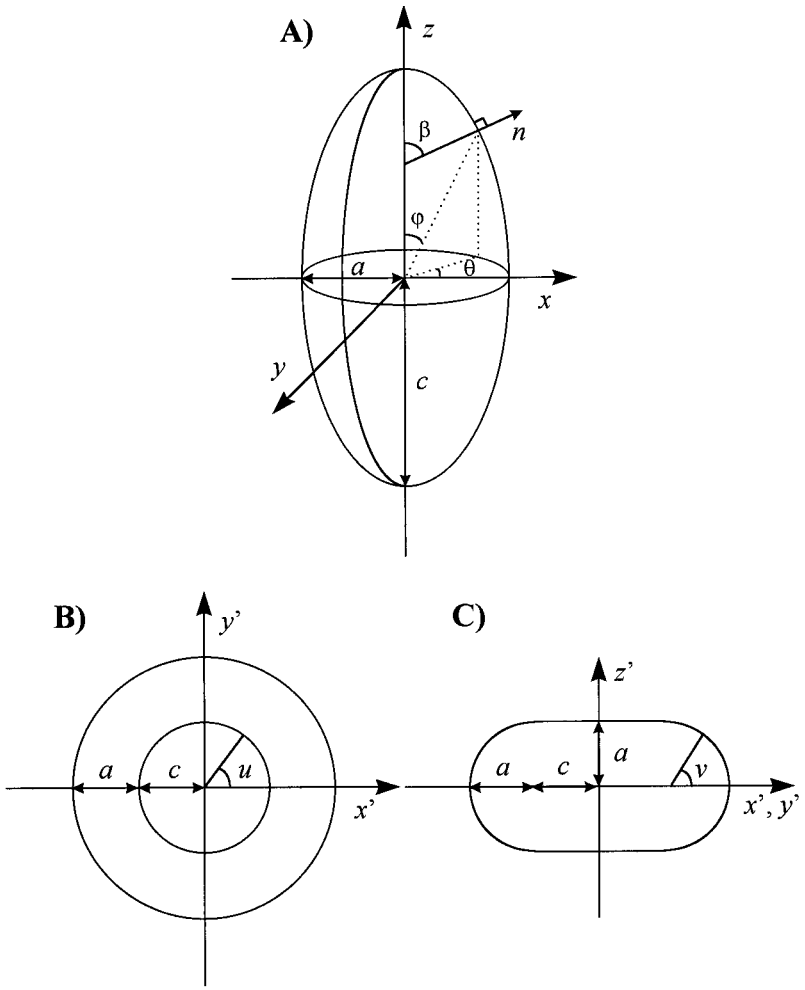

FIGURE 1 Geometrical representations of (*A*) an ellipsoid, (*B*) a bicelle in the x'–y' plane, and (*C*) a bicelle in the $(x'y')$–z' plane.

an oblate spheroid if c is smaller than a. It can be seen in Fig. 1 that, even if the angle that determines the NMR frequency is β (i.e., the angle between the axis of motional averaging and the magnetic field), the ellipsoid is defined in terms of φ and θ. Therefore, it is important to relate the distribution of probability expressed in terms of φ and θ to the distribution of probability expressed in terms of β.

The tangents to the surface of the ellipsoid, $\vec{n}_\theta$ and $\vec{n}_\varphi$, can be obtained by the partial derivation of Eq. 4 relative to φ and θ,

$$\vec{n}_\varphi = \begin{bmatrix} a\cos\theta\cos\varphi \\ a\sin\theta\cos\varphi \\ -c\sin\varphi \end{bmatrix}, \tag{5}$$

$$\vec{n}_\theta = \begin{bmatrix} -a\sin\theta\sin\varphi \\ a\cos\theta\sin\varphi \\ 0 \end{bmatrix}. \tag{6}$$

Using the normalized cross product of the two tangents, a normal unit vector is obtained,

$$\vec{n} = \frac{\vec{n}_\varphi \times \vec{n}_\theta}{\|\vec{n}_\varphi \times \vec{n}_\theta\|} = \frac{1}{\sqrt{a^2\cos^2\varphi + c^2\sin^2\varphi}} \begin{bmatrix} c\cos\theta\sin\varphi \\ c\sin\theta\sin\varphi \\ a\cos\varphi \end{bmatrix}. \tag{7}$$

The projection of the normal unit vector along z is related to β via the equation,

$$\vec{n}\cdot\vec{k} = \cos\beta = \frac{a\cos\varphi}{\sqrt{a^2\cos^2\varphi + c^2\sin^2\varphi}}. \tag{8}$$

Rearranging this equation, it is found that φ and β are related via the equation,

$$\tan\varphi = \frac{a}{c}\tan\beta. \tag{9}$$

The angular distribution is related to a surface element, ds, which is given by

$$\begin{aligned} ds &= \|\vec{n}_\varphi\|\cdot\|\vec{n}_\theta\|\cdot \mathrm{d}\varphi\mathrm{d}\theta \\ &= a\sin\varphi\sqrt{a^2\cos^2\varphi + c^2\sin^2\varphi}\,\mathrm{d}\varphi\mathrm{d}\theta. \end{aligned} \tag{10}$$

An integration over θ and the use of Eq. 9 gives the angular distribution of an ellipsoid,

$$P_E(\beta) = \frac{2\pi c^2\sin\beta}{[\sin^2\beta + r^2\cos^2\beta]^2}, \tag{11}$$

where $r = c/a$. Then, the lineshape of an ellipsoid, prolate or oblate, can be determined using Eqs. 1, 3, and 11,

$$S_E(\omega) = \frac{2\pi c^2 (3\delta)^{3/2}}{\sqrt{2\omega + \delta}[(2\delta - 2\omega) + r^2(2\omega + \delta)]^2}. \quad (12)$$

Oriented bicelles

In this model, the outer part of a bicelle is a torus and the inner part is a circular plane. A representation of a bicelle can be seen in Fig. 1. These two parts of the bicelle will be treated separately. The parametric equations of a torus are

$$x' = (c + a\cos v)\cos u,$$

$$y' = (c + a\cos v)\sin u,$$

$$z' = a\sin v, \quad (13)$$

where the radii a and c and the angles u and v are defined in Fig. 1. The tangents to the surface of the torus, $\vec{n}_u$ and $\vec{n}_v$, can be obtained by the partial derivation of Eq. 13 relative to u and v,

$$\vec{n}_u = \begin{bmatrix} -(c + a\cos v)\sin u \\ (c + a\cos v)\cos u \\ 0 \end{bmatrix}, \quad (14)$$

$$\vec{n}_v = \begin{bmatrix} -a\sin v\cos u \\ -a\sin v\sin u \\ a\cos v \end{bmatrix}. \quad (15)$$

Using the normalized cross product of the two tangents, a normal unit vector is defined,

$$\vec{n} = \frac{\vec{n}_u \times \vec{n}_v}{\|\vec{n}_u \times \vec{n}_v\|} = \begin{bmatrix} \cos v\cos u \\ \cos v\sin u \\ \sin v \end{bmatrix}. \quad (16)$$

The lineshape for a bicelle oriented with its main axis (i.e., the normal vector to the bilayer plane) parallel to the magnetic field will first be derived in this section. To do so, we proceed as for the ellipsoidal orientation by taking the scalar product of the normal unit vector and the unit vector along z,

$$\vec{n} \cdot \vec{k} = \cos\beta = \sin v. \quad (17)$$

This equation gives directly the relationship between v and β. Then, the surface area element is obtained from the tangents,

$$ds = \|\vec{n}_u\| \cdot \|\vec{n}_v\| \cdot \mathrm{d}u\mathrm{d}v = a(c + a\cos v)\mathrm{d}u\mathrm{d}v. \quad (18)$$

After an integration over u and using Eq. 17, the density of probability of the torus is found,

$$P''_t(\beta) = 2\pi a(c + a\sin\beta). \quad (19)$$

Then, the lineshape of the outer part of a torus oriented with its principal axis along z is determined using Eqs. 1, 3, and 19,

$$S''_t(\omega) = \frac{2\pi r(1 + r\sqrt{(2\delta - 2\omega)/3\delta})}{\sqrt{(2\delta - 2\omega)(2\omega + \delta)}}, \quad (20)$$

where $r = a/c$. Finally, the total lineshape of the bicelle is

$$S''_B(\omega) = \rho\frac{S''_t(\omega)}{\int S''_t(\omega)\,\mathrm{d}\omega} + (1 - \rho)\frac{S''_p}{\int S''_p(\omega)\,\mathrm{d}\omega}, \quad (21)$$

where the lineshape associated to the planes can be considered as a delta function $S''_p(\omega) = \boldsymbol{\delta}(\omega - \delta)$. The relative proportion of the torus spectrum in the bicelle spectrum is defined as the area of the outer part of a torus over the total area of the bicelle,

$$\rho = \frac{r(\pi + 2r)}{r(\pi + 2r) + 1}. \quad (22)$$

When fast motions relative to the ^{31}P NMR time scale occur, such as the rotation of the bicelle around its main symmetry axis and the lateral diffusion of the lipids located in the edge section of the bicelles, there is no more frequency distribution. In such cases, the weighted-average frequency can be calculated,

$$\langle\omega\rangle = \left(\frac{\delta}{2}\right)\frac{\int_0^{\pi/2}(3\cos^2\beta - 1)P(\beta)\,\mathrm{d}\beta}{\int_0^{\pi/2}P(\beta)\,\mathrm{d}\beta} + \delta_{iso}. \quad (23)$$

For a bicelle oriented with its principal axis perpendicular to z,

$$\vec{n} \cdot \vec{i} = \cos v\cos u = \cos\beta. \quad (24)$$

This equation does not give a direct relationship between two angles and, therefore, it is not possible to use the same approach as that used for the bicelle oriented with its normal parallel to the magnetic field. However, using a delta function, a surface element $\mathrm{d}s'$ can be defined as

$$\mathrm{d}s' = \left\{\int \boldsymbol{\delta}(\cos u\cos v - \cos\beta)\right\}\mathrm{d}s \cdot d\cos\beta, \quad (25)$$

which becomes

$$\mathrm{d}s' = 2\pi a^2\sin\beta\mathrm{d}\beta + 4ac\left\{\int_0^\beta \frac{\mathrm{d}v}{\sqrt{1 - \csc^2\beta\sin^2 v}}\right\}\mathrm{d}\beta. \quad (26)$$

When $c \to 0$, this surface element corresponds to the surface element of a sphere. Unfortunately, this equation cannot be solved because it contains an elliptic integral. However, because perpendicular bicelles spectra can be almost perfectly simulated by considering the presence of lateral diffusion and tumbling as demonstrated in the Result section, the static perpendicular bicelle spectra were not simulated here. In the presence of rapid motions such as tumbling and

lateral diffusion, the weighted-average frequency obtained for perpendicular bicelles can be calculated using Eq. 23 and multiplied by a S_{tilt} value of -0.5, as described below.

Order parameters S_{dist} and S_1

As discussed above, if there is a change in the shape of the orientation distribution, there is also a change in the weighted-average frequency. Rearranging Eq. 23, it is possible to define a distribution order parameter,

$$S_{dist} = \frac{\langle\omega\rangle - \delta_{iso}}{\delta}. \tag{27}$$

It should also be mentioned that the weighted-average frequency is exactly the same as a first moment calculation, i.e., $M_1 = \langle\omega\rangle$. Therefore, a calculation of the spectral moments could be a measure of the orientation. Obviously, this equation is only valid if the main axis of the distribution is along z. If the system main axis is tilted relative to the magnetic field axis, a new total order parameter can be introduced,

$$S_1 = S_{dist} \cdot S_{tilt}. \tag{28}$$

This new order parameter is denoted S_1 to emphasize that it comes experimentally from a first moment calculation. It is possible to solve these order parameter equations for many systems by considering their angular distributions. For a spherical vesicle, $S_1 = 0$. In the case of an ellipsoid, assuming that the main axis is along z, the order parameter S_1 is

$$S_1 = \frac{3}{2(r^2-1)}\left\{\frac{A-1}{A+1}\right\} - \frac{1}{2}. \tag{29A}$$

If $r < 1$,

$$A = \frac{r^2}{2\sqrt{1-r^2}} \ln\left\{\frac{1+\sqrt{1-r^2}}{1-\sqrt{1-r^2}}\right\}, \tag{29B}$$

whereas, if $r > 1$,

$$A = \frac{r^2}{\sqrt{r^2-1}} \arctan\{\sqrt{r^2-1}\}. \tag{29C}$$

For bicelles, because their main axis can be oriented at different angles relative to the external magnetic field, it is more appropriate to define a distribution order parameter using the distribution of orientation and Eq. 22,

$$S_{dist} = \frac{1}{4}\left(\frac{\pi r + 4}{\pi r + 2r^2 + 1}\right). \tag{30}$$

Then, the S_1 order parameter is determined from Eq. 28 using an S_{tilt} value of 1 for parallel bicelles and -0.5 for perpendicular bicelles. It is also possible to use any other S_{tilt} values for partially tilted bicelles.

MATERIAL AND METHODS

Material

DMPC, DHPC, and CL were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification. GA and ADM (doxorubicin hydrochloride) were obtained from Fluka (Ronkonkoma, NY) and used without further purification.

Sample preparation

Aqueous dispersions of DMPC and CL were prepared in a 150 mM NaCl and 10 mM EDTA solution and adjusted to pH 6.5. Samples containing 20% (wt/wt) of lipids were then heated to ~50°C for 10 min, stirred on a vortex mixer, and cooled down at 0°C for 10 min. This cycle was repeated at least five times just before the analysis. The solution of ADM (0.01 g/mL) was prepared in 150 mM NaCl and 10 mM EDTA and adjusted to pH 6.5. The appropriate volume of the ADM solution was added to the CL dispersion to obtain a 2:1 CL:ADM molar ratio and then, five freeze–thaw cycles were applied to the system. Samples of 2.7:1 DMPC:DHPC molar ratio were prepared in a 0.1 M KCl buffer. Then samples containing 20% (wt/wt) of lipids were heated to ~35°C for 10 min, stirred on a vortex mixer, sonicated, and cooled down at 0°C for 10 min. This cycle was repeated at least five times just before the analysis. Samples of 10:4.3:1 DMPC:DHPC:GA molar ratio were prepared in trifluoroethanol. To obtain homogeneous peptide/lipid systems, the samples were incubated at 52°C for 1 h and shaken on a vortex mixer at least a few times during the incubation cycle. After the incubation, the organic solvent was evaporated with a nitrogen stream followed by high vacuum pumping overnight. The samples were then hydrated at 32% (wt/wt) with a HEPES buffer prepared at pH 7.0 and were submitted to several cycles of heating (52°C), vortex-mixer, sonication, and cooling (0°C).

NMR experiments

The ^{31}P NMR spectra were acquired at 121.5 MHz on a Bruker ASX-300 (Bruker Canada Ltd., Milton, ON) operating at a ^{1}H frequency of 300.0 MHz. Experiments were carried out with a broadband/^{1}H dual frequency 4-mm probehead. The free induction decays (2 K data points) were recorded with a spin echo sequence (2000 or 4000 scans) with a 4 to 7 s repetition time and under conditions of proton decoupling. The ^{1}H 90° pulse length was typically 4.0 μs, corresponding to a rotating-frame frequency of about 63 kHz. The ^{31}P 90° pulse length was 5 μs and the interpulse delay was set to 30 μs to avoid anisotropic T_2 effects on static spectra. The temperature was controlled to within ±0.5°C and the chemical shifts expressed in parts per millions (ppm) were referenced relative to the signal of phosphoric acid at 0 ppm. When not specified, a line broadening of 50 Hz was applied to the spectra. Magic angle spinning (MAS) ^{31}P NMR spectra were obtained with a spinning speed of 6 kHz and a free induction decay of 4 K data points (1000 scans). No line broadening was applied to these spectra.

Simulations and calculations

Simulations and calculations were performed with the Grams 386 software (Galactic Industries Corp., Salem, NH) using the Array Basic programming language. The simulated spectra were broadened by convolution with a Gaussian function. The experimental spectral treatment was done using the UXNMR software from Bruker (Bruker Canada Ltd., Milton, ON).

RESULTS AND DISCUSSION

Theoretical spectra

In this section, we will present the simulated ^{31}P NMR spectra for partially oriented membranes, using the formal-

ism developed in the Theory section. The simulated spectra will then be used to obtain the order parameters S_1 and S_2, respectively related to the orientation and the dynamics of membranes.

Figure 1 shows two types of partially oriented membranes, an ellipsoidal vesicle in *A* and two views of a bicelle in *B* and *C*. If partial orientation occurs in model membranes, they will most likely adopt an ellipsoidal structure in which *a* will be smaller than *c* due to the sign of the lipid diamagnetic anisotropy (Boroske and Helfrich, 1978). In this kind of system, the majority of the phospholipids will be oriented with their main axis, $\vec{n}$, at 90° relative to the surface. Such an ellipsoidal orientation implies either very large unilamellar or multilamellar vesicles because phospholipids in an ellipsoid with a very short semi-axis *a* will experience a fast angular diffusion that would result in chemical shifts characteristic of a hexagonal phase rather than the ones characteristic of lamellar phases (Seelig, 1978).

Figure 2 shows the simulated spectra of ellipsoidal structures for *r* ranging from 5 to 0.2. At a ratio of 5, the phospholipids are almost all oriented at 90° relative to the magnetic field. The ratio of 1 corresponds to a spherical vesicle, and, at a ratio of 0.2, the majority of the phospholipids is oriented parallel to the magnetic field. The surprising point of these simulated spectra is that a prolate ellipsoid gives a very small distribution of frequencies in comparison with the broad distribution occurring for an oblate shape. This can be seen in Fig. 2 by comparing the spectra with $r = 2.5$ and 0.4.

The second case of partial orientation is the bicelle, which is usually made of a long acyl chain phospholipid that has been assumed to be on the two circular planes, and of a short acyl chain phospholipid that has been assumed to be on the rim of the bicelle (Sanders and Schwonek, 1992; Vold and Prosser, 1996). Bicelles made of only two circular planes have also been proposed for systems composed of CHAPSO and DMPC (Sanders and Prestegard, 1990; Sanders et al., 1994). In the present study, the bicelles are modeled by the combination of the outer part of a torus and of two circular planes (Fig. 1, *B* and *C*). Simulations will be made using two different models. In the first model, we will consider a constant molecular surface area, a constant composition of phospholipids over the whole surface of the bicelle and a negligible contribution of the lipid lateral diffusion. In the second model, we will consider a rapid axial rotation of the bicelles around their main axis in addition to a rapid lateral diffusion of the lipids. In these two models, the bicelles are considered to be made of a single bilayer in which the thickness is the double of the radius of the edge, as illustrated in Fig. 1 *C*. The axis system of the bicelles is denoted (x', y', z') to account for the difference with the laboratory frame (x, y, z). Two different orientations of the main axis of the bicelles are modeled, a bicelle with its z' axis along z and a bicelle with its x' axis along z.

Figure 3 *A* reports the simulation of ^{31}P NMR spectra of static parallel bicelles in which the effect of the orientation can be easily seen as an increasing spectral component at $\delta_{\|}$. An important point to note is that a highly oriented system, e.g., with an *r* value of 0.5 corresponding to a bicelle thickness of 50 Å and to a bicelle total diameter of 200 Å, gives a broad spectrum if the main axis of the bicelle is oriented parallel to the magnetic field. However, because the broad component is due to the rim of the bicelle, static parallel bicelles should give sharp NMR peaks for molecules exhibiting fast axially symmetric motions inserted into

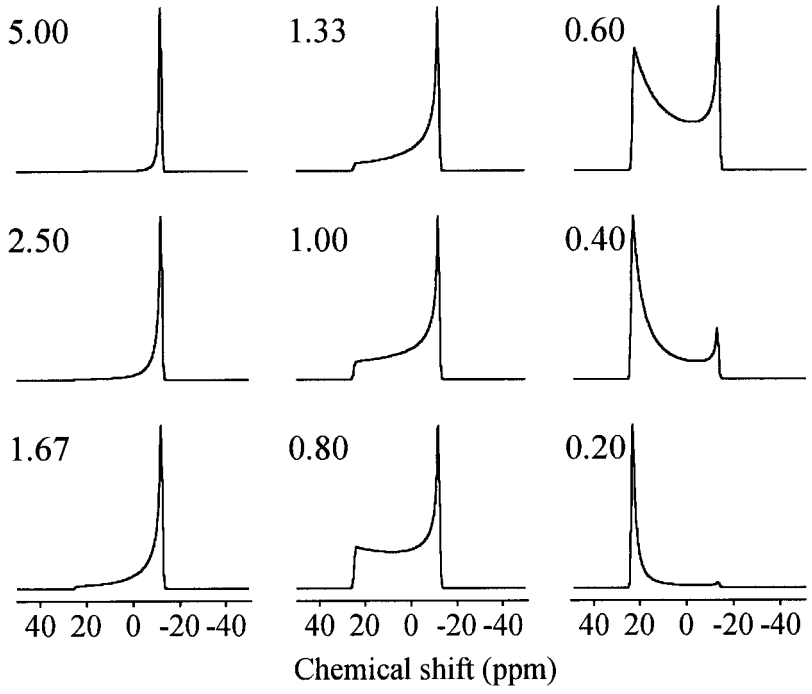

FIGURE 2 ^{31}P NMR spectral simulations of ellipsoidal vesicles as a function of *r* with $\delta = 25$ ppm, $\delta_{iso} = 0$ ppm and a Gaussian broadening (FWHH = 1 ppm).

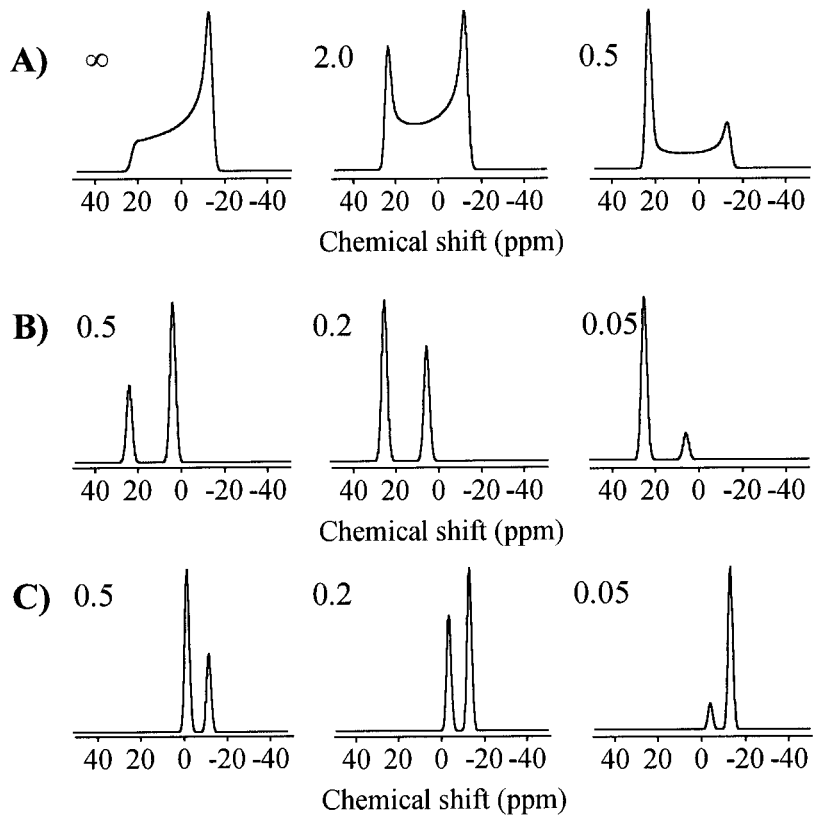

FIGURE 3 ^{31}P NMR spectral simulations of bicelles as a function of r with δ = 25 ppm, δ_{iso} = 0 ppm and a Gaussian broadening (FWHH = 3 ppm). (*A*) Static parallel bicelles. (*B*) Parallel bicelles with axial tumbling and lateral diffusion. (*C*) Perpendicular bicelles with axial tumbling and lateral diffusion.

their flat sections, such as membrane proteins. Finally, the spectral difference between parallel bicelles and oblate spheroids allows a discrimination of the two types of organization, as discussed in a later section.

Figure 3, *B* and *C*, shows simulated parallel and perpendicular bicelle spectra in which tumbling and lateral diffusion have been considered. In this model, we supposed that the phospholipids that constitute the torus do not diffuse in the planes and vice-versa. This assumption has been made because experimental bicelles are constituted of two types of phospholipids that are believed to be laterally phase separated. The relative proportion of the two peaks observed in these spectra varies with the r shape parameter. This relationship is defined by Eq. 22 and is plotted in Fig. 4 *A*. Therefore, from an experimental spectrum, it is possible to determine the size of the bicelle only by integrating the two peaks. The frequency of the peak from the torus part can also be related to the r shape parameter by

$$\langle \omega_{torus} \rangle = \frac{\pi}{4\pi + 8r}. \tag{31}$$

An S_{dist} order parameter can be calculated from this weighted-average frequency using Eq. 27. This relationship, plotted in Fig. 4 *A*, could also be a measure of the r shape parameter. A value of 0.25 for the center of gravity of the torus section of bicelles relative to the bilayer normal has already been proposed in the literature (Vold and Prosser, 1996) for ^{2}H NMR spectra.

As discussed before, in oriented systems, it is possible to define an order parameter from the first spectral moment measured experimentally, which will be a measure of the orientation level. A system with a value of S_1 close to 1 (-0.5) indicates that all the molecules are oriented with their main axis parallel (perpendicular) to the magnetic field. Figure 4 *B* reports the variation of S_1 with the shape parameter r assuming that the main axis of the oriented system is along z for ellipsoids and either along or perpendicular to z for bicelles.

A relative order parameter can also be defined, namely the ratio of the spectral widths of an experimental spectrum to that of a reference spectrum,

$$S_2 = \frac{\delta}{\delta^{ref}}. \tag{32}$$

This order parameter, denoted S_2 to emphasize that it is related to the second spectral moment, can vary from 1 for a system in which the dynamics are the same as those in a reference system to 0 if the system becomes totally isotropic. Several reference systems can be chosen, such as the CSA of DMPC multilamellar vesicles when investigating bicelles made of DMPC and DHPC. This order parameter combines the effects of two other order parameters, a first one that has already been introduced in the literature as $S_{bilayers}$ (Sanders and Schwonek, 1992), which denotes the presence of local motional averaging of the CSA, and a second that we have previously named S_{tilt} and that some

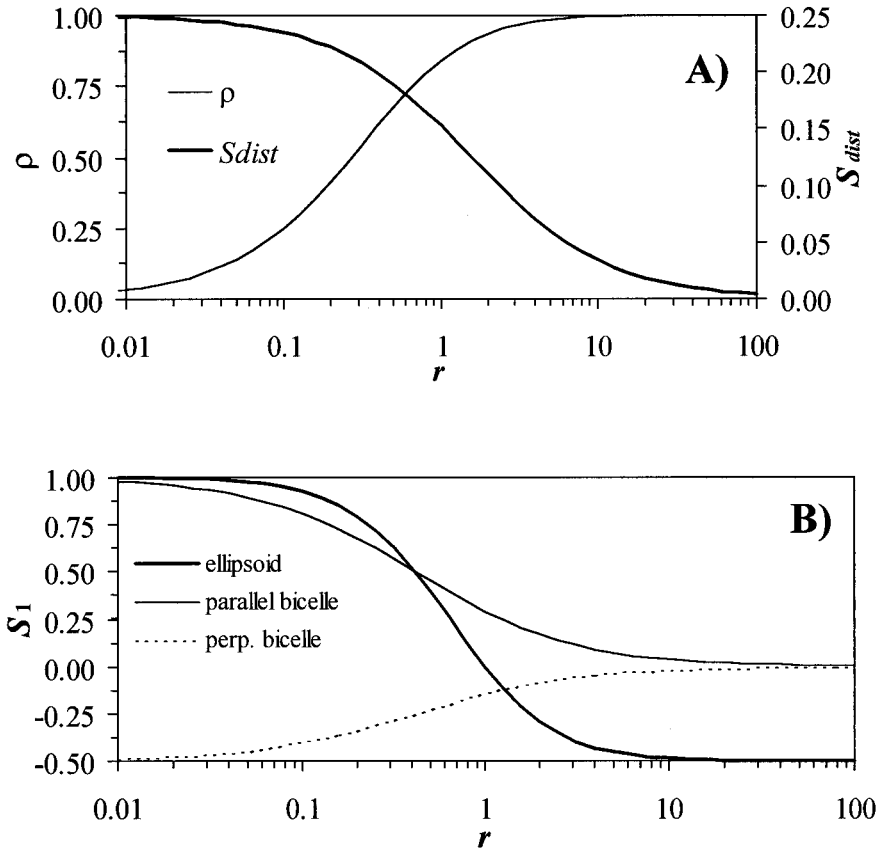

FIGURE 4 (*A*) Relative proportion, ρ, and S_{dist} for the lipids in the torus part of the bicelles. (*B*) S_1 order parameter for ellipsoidal vesicles, parallel bicelles, and perpendicular bicelles as a function of the shape parameter r.

authors have introduced as S_{system} (Sanders et al., 1994), which relates the orientation of the main axis of the system to the magnetic field axis. However, in the present study, the main axis of each system is considered to be oriented either parallel or perpendicular to z, which implies that a change in S_2 is considered as a change in the dynamic properties (lateral diffusion, wobbling, tumbling) of the lipids rather than an orientational change of the main axis of the oriented system (such as a tilt of the bicelles).

Experimental systems

To validate the spectral simulations and the use of the order parameters presented in the previous section, several partially oriented lipid systems have been investigated. More specifically, we have first investigated the partial orientation of pure DMPC multilamellar vesicles as a function of temperature and of hydration level. This system is known to be partially deformed in high magnetic fields, and the results of the present study will show that the partial orientation can be quantitatively defined by the order parameter S_1. A similar approach will then be applied to DMPC:DHPC bicelles, a lipid system known to orient with the lipids oriented at 90° relative to the magnetic field. Finally, we will investigate two cases of partial parallel lipid orientation, namely the parallel orientation of DMPC:DHPC membranes in the presence of GA and the partial orientation of CL membranes in the presence of ADM.

Pure DMPC

Figure 5 *A* presents the ^{31}P NMR spectra of DMPC recorded at different conditions of concentration and temperature to study their partial orientation in the magnetic field. These spectra are characteristic of phospholipids in the liquid-crystalline phase. At a concentration of 40%, the spectrum is very close to the spectrum of a spherical distribution. However, lowering the concentration results in a decrease of the intensity of the lipids oriented at 0° relative to the magnetic field, which confirms the presence of partial orientation. It is also possible to note the appearance of an isotropic peak, which could be related to the formation of smaller structures in which the tumbling and the lateral diffusion correlation times become on the same order as the NMR acquisition time. Even if hydration seems to have the most important effect on the partial orientation, temperature also seems to induce a change of the orientation distribution.

These spectral deformations were investigated by an orientation calculation. Information about the orientation in this system could be obtained only if the isotropic and anisotropic chemical shifts are evaluated precisely. The isotropic chemical shifts were obtained from MAS spectra but are not reported here, and the chemical shift anisotropy was evaluated directly on the static spectra. The CSA can be obtained in different ways, such as evaluating the second spectral moment. However, simulations have proven that an extremely accurate measurement of the CSA is not neces-

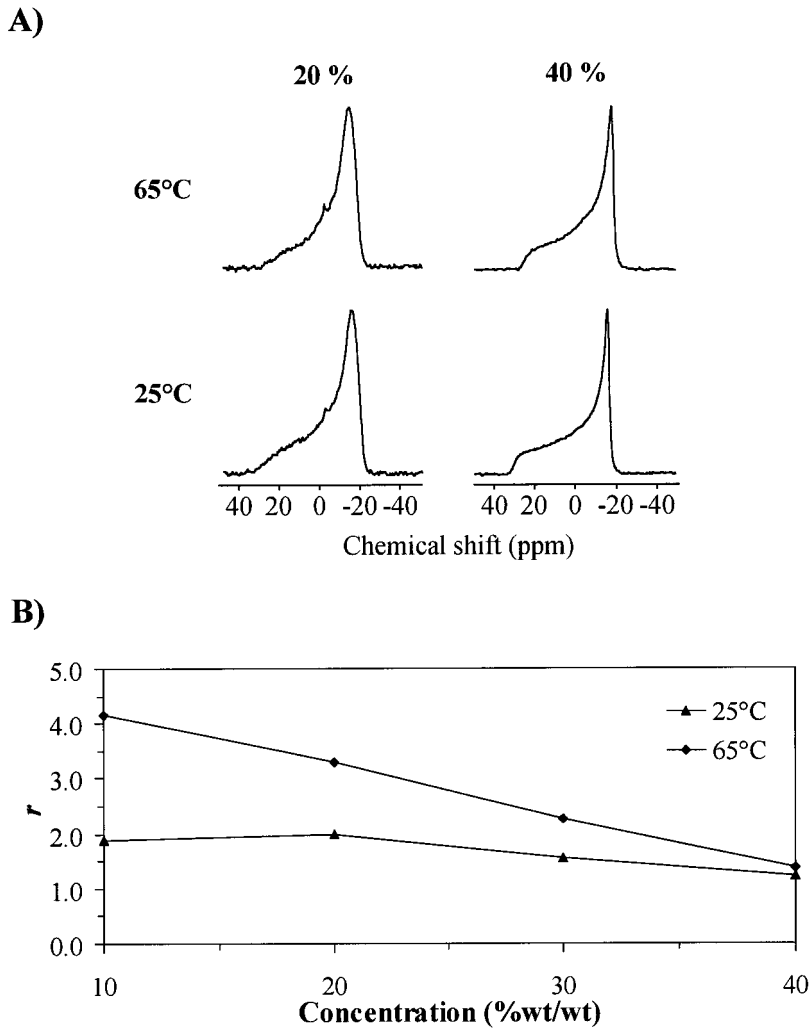

FIGURE 5 (*A*) Experimental ^{31}P NMR spectra. (*B*) *r* Shape parameter for DMPC as a function of temperature and concentration (wt/wt %).

sary for the orientational calculations (results not shown) and we have therefore evaluated this parameter by taking the chemical shift at 90% of the maximum spectral intensity. Our results indicate that both the CSA and the isotropic chemical shift vary linearly with temperature, with a transition at the gel to liquid-crystalline phase transition (results not shown). Then, an ellipsoidal parameter, *r*, was evaluted from the first spectral moment using Eq. 29 and plotted as a function of the phospholipid concentration for two temperatures in Fig. 5 *B*. The ratio of the semi-axes *c*/*a* increases with temperature and DMPC concentration, indicating a higher orientation at high temperature and low concentration. This is related to the membrane elasticity, which increases with an increasing temperature and decreasing concentration.

Bicelles

The second system investigated in the present study is made of DMPC and DHPC at a molar ratio of 2.7:1. This system is supposed to form discoidal structures that orient their main axis at 90° relative to the magnetic field. Figure 6 *A* shows the spectra obtained as a function of temperature. Below 25°C, the spectrum is composed of an isotropic peak that can be attributed to DHPC and of a broad spectrum that can be attributed to DMPC (Sanders and Schwonek, 1992). The intensities of the two subspectra correspond approximately to the molar fraction of DMPC and DHPC used in the sample preparation. From 25° to 35°C, the spectra indicate that the system becomes more and more oriented, i.e., the intensity corresponding to a perpendicular orientation of the phospholipids increases greatly. A second smaller peak, characterized by a different chemical shift, is also present and its chemical shift decreases with temperature.

In a previous section, we have demonstrated that, as some authors have suggested (Sanders and Schwonek, 1992; Vold and Prosser, 1996), the smaller peak is due to phospholipids in the edges of the bicelle, most likely the short-chain lipid DHPC. However, the relative intensity of the two peaks changes with time, which indicates that these two peaks might not be solely attributed to DMPC and DHPC. At temperatures above 40°C, the smaller peak disappears at the expense of an isotropic peak. This corresponds to a well-known property of bicelles, namely the existence of a

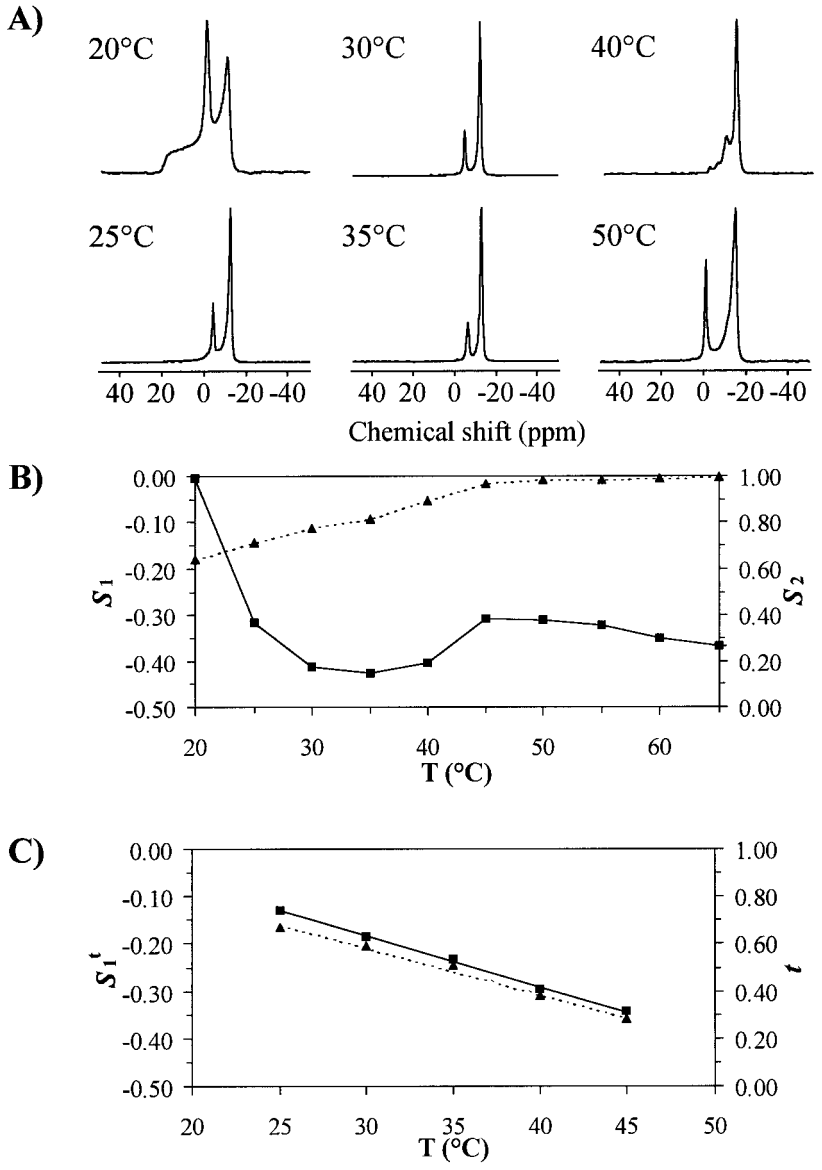

FIGURE 6 (*A*) Experimental ^{31}P NMR spectra. (*B*) S_1 and S_2 order parameters and (*C*) S_1^t order parameter and t for DMPC:DHPC (2.7:1 molar ratio) as a function of temperature.

magnetic phase transition at 40°C (Sanders and Schwonek, 1992). At temperatures above 40°C, the proportion of phospholipids with a 90° orientation seems to increase with temperature and the intensity of the isotropic peak decreases.

The order parameters S_1 and S_2 have been calculated for these spectra using pure DMPC multilamellar vesicles as the reference system. More specifically, S_1 is calculated from the first spectral moment. In a previous section, we have demonstrated that, even in the presence of two distinct peaks, S_1 can be calculated if the peaks have the same isotropic chemical shift. This seems very likely because DMPC and DHPC have the same headgroup. In addition, even if the two peaks are not solely attributed to DMPC or DHPC, no fine structure is observed in the two peaks, again suggesting similar isotropic chemical shifts and CSA for the two lipids. Figure 6 *B* shows the variation of S_1 as a function of temperature. S_1 has a value of 0 at 20°C, representing no orientation and, as the temperature increases, S_1 decreases to -0.45 at 35°C, representing an almost complete perpendicular orientation of the lipids. Then, S_1 increases to -0.35 at 50°C and decreases to -0.40 at 65°C. The greatest orientation is therefore at 35°C with a transition between 40 and 50°C. These results indicate that bicelles are well oriented between 30 and 40°C. This temperature range is similar to that obtained by other groups (Sanders and Schwonek, 1992; Losonczi and Prestegard, 1998; Ottiger and Bax, 1998) that demonstrated that bicelles are well oriented between 30 and 40–45°C. The slight difference observed in the upper limit temperature might be due to a slight sampling heating effect resulting from the high proton decoupling power used in the present study.

The results presented above indicate that S_1 is a good tool for representing the magnetic orientation of phospholipids because it is directly related to the shape of the membrane. Another order parameter that can be measured on these

spectra is S_2, which is related to the dynamics in the system relative to a reference system. In this case, the reference system is pure DMPC. S_2 is plotted as a function of temperature in Fig. 6 *B*. In the supposed temperature range of existence of the bicelles (<40°C), the order parameter indicates that the system is less ordered than is pure DMPC. This is in agreement with the formation of small structures such as bicelles. At high temperatures, S_2 is equal to 1, indicating that the averaging of the chemical shift tensor is similar to that obtained for pure DMPC. The spectra are also broader and S_1 higher, showing that the system is most likely constituted of bigger structures less oriented than the bicelles at low temperature. These comments are based on the assumption that the S_{tilt} of the bicelles equals −0.5. In this system, the parameter S_2 therefore provides important and complementary information about the dynamics in the system.

To go further in the analysis of the system, we have used the proposed shape of the bicelles discussed in the Theory section to obtain the r (a/c ratio) value from the relative proportion of the two peaks at 35°C. Using Eq. 22, we found a value of 0.07, indicating that the semi-axis c is 15 times the value of the semi-axis a. Assuming that the bicelle thickness is 50 Å, the total diameter of the bicelle is ~750 Å. In contrast, it is possible to determine the r shape parameter from the total S_1 value at 35°C. Using Eqs. 28 and 30, we found that $r = 0.05$, indicating that the semi-axis c is 20 times the value of the semi-axis a, which gives a bicelle diameter of ~1000 Å. Therefore, these two different methods give approximately the same diameter, which corresponds to the proposed diameter for discoidal structures in lyotropic liquid-crystals (Forrest and Reeves, 1981). Another way to determine the r shape parameter is from the S_1 of the edge phospholipids. This value is plotted as a function of temperature in Fig. 6 *C*. The first remarkable feature is the linear relationship between S_1 and temperature between 25° and 45°C. However, it is surprising that all the S_1 values are below −0.125, the limit value. A way to explain this phenomenon is to suppose an exchange between the phospholipids in the torus and in the planes of the bicelles. The resulting order parameter could be represented by

$$S_1 = t \cdot S_1^t + (1 - t) \cdot S_1^p. \quad (33)$$

In this equation, t represents the proportion of time spent by a phospholipid in the torus. This calculation has been performed on the data presented in Fig. 6 *A* and the results are plotted in Fig. 6 *C*. These results show that the phospholipid lateral diffusion becomes more important with increasing temperature. In addition, they suggest that the phospholipids in the edges of the bicelle are partially phase separated from the lipids in the planes, but that a fast exchange process exists between them. Vold and Prosser (1996) have investigated the same system by ^{2}H NMR and showed, using labeled DHPC, that this phospholipid is located solely in the edges of the bicelle. This difference can be easily explained by the longer timescale of ^{31}P NMR relative to ^{2}H NMR.

Parallel lipid orientation

One potential limitation in using the magnetically oriented bilayers described in the two previous sections for structural studies is that, because this system is characterized by a negative orientational order parameter ($S_1 \approx -0.5$), a well-resolved NMR spectrum with sharp lines will only be obtained if the molecule of interest undergoes fast axially symmetric motions. Otherwise, the NMR spectra will exhibit cylindrical powder patterns (Prosser et al., 1998). For this reason, the alignment of phospholipid bilayers with their director parallel to the magnetic field (i.e., with $S_1 = 1$) has been the goal of several research efforts. Recently, it was observed that the addition of small amounts of paramagnetic ions such as Eu^{3+} or Yb^{3+} to DMPC/DHPC bicelles results in systems in which the director is oriented parallel to the magnetic field (Prosser et al., 1996). Another way to obtain a parallel lipid orientation without these paramagnetic effects would be the addition of a molecule (protein, peptide, or drug) with a large positive $\Delta\chi$ (Sanders et al., 1993). This avenue was used in the present study. More specifically, we have first prepared bicelles in the presence of the transmembrane peptide GA due to its high content in aromatic residues and to its helical conformation. Figure 7 *A* shows the spectra of DMPC:DHPC:GA as a function of temperature and time. These spectra clearly show the appearance of a spectral component at $\delta_\parallel$ both with increasing temperature but also as a function of the time spent in the magnet.

The comparison between the spectra presented in Fig. 7 *A* and the simulated spectra presented in Figs. 2 and 3 indicates that the shape of the partially oriented system is closer to a static parallel bicelle than to a parallel ellipsoid. To obtain more quantitative information about this orientation phenomenon, S_1 and S_2 were calculated using pure DMPC multilamellar vesicles as the reference system. The S_2 value is constant at 0.7 for all spectra. Thus, it can be considered that these systems are less ordered than pure DMPC. This is in agreement with the disordering effect also observed in ^{31}P NMR spectra of unoriented DMPC lamellar vesicles in the presence of GA (Bélanger, A. and Auger, M., unpublished results). Fig. 7 *B* presents the S_1 calculation that provides quantitative information about the orientation of the lipid systems. Hence, S_1 goes up to 0.55 in the last spectrum, which is representative of a high orientational order. In addition, Fig. 7 *B* shows the r (a/c) ratio obtained from the order parameter S_1. For the highly oriented system at the end of the experiment, the calculation of the diameter from the r value indicates that the bicelle has a diameter four times bigger than its thickness. This observation is in agreement with the proposed size of bicelles (Vold and Prosser, 1996).

Parallel lipid orientation without DHPC? the CL:ADM complex

Another oriented system with the lipids parallel to the magnetic field is the CL:ADM complex at a 2:1 molar ratio.

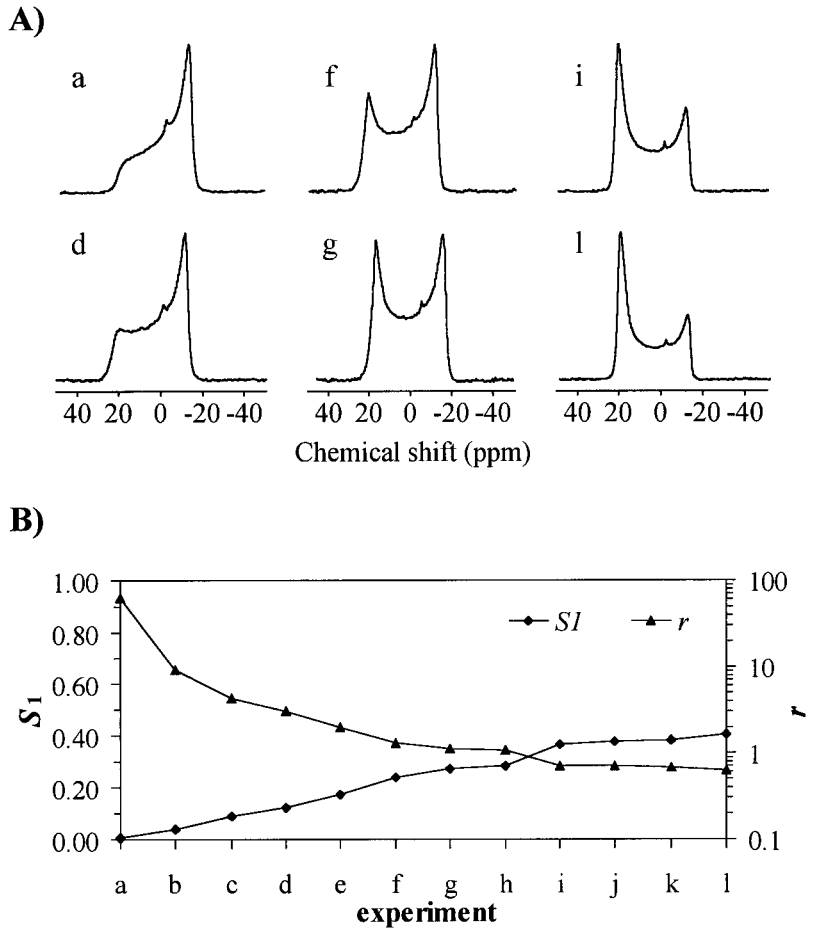

FIGURE 7 (*A*) Experimental ^{31}P NMR spectra and (*B*) S_1 and r for DMPC:DHPC:GA (10:4.3:1 molar ratio) as a function of temperature and time. The spectra have been recorded every four hours at the following temperatures: (*a*) 25°C, (*b*) 30°C, (*c*) 35°C, (*d*) 40°C, (*e*) 45°C, (*f* to *l*) 50°C.

ADM is a highly aromatic molecule used currently in chemotherapy as an antineoplastic agent. This molecule is known to interact strongly with negatively charged lipids such as CL, a phospholipid found in the negatively charged cardiac cellular membranes. ADM has been shown to cause cardiac arrest if taken at high dosage. A model of interaction for this system has been proposed in the literature in which the ADM molecules are stacked on the membrane at an angle of 39° relative to the bilayer normal (Goormaghtigh et al., 1987). This arrangement is characterized by a large value of $\Delta\chi$ which favors its magnetic orientation. Figure 8 *A* shows the spectra of the CL:ADM complex at a 2:1 molar ratio as a function of temperature. Significant magnetic orientation occurs in this system as the temperature increases, which is in agreement with the model of interaction proposed for this complex. Comparing these lineshapes with those presented in Figs. 2 and 3 indicates that these spectra could again be associated to a bicellar organization. However, the resolution is not as good and the spectrum is broader, characteristic of a distribution of CSA. Another satisfactory model could be an intermediate system composed of spherical vesicles with flat sections where the phospholipids are oriented parallel to the magnetic field.

Figure 8 *B* shows the results of the order parameter calculations for the ADM:CL system. The value of S_1 increases gradually with temperature. However, the orientation does not become as high as that observed in the DMPC:DHPC:GA system. Using pure CL multilamellar vesicles as the reference system, the value of S_2 is ~1 at all temperatures, indicating that the dynamics in this system are close to those of the pure CL system. These observations are in agreement with a broader distribution of orientations that is probably not associated to a perfect bicellar system. The r shape parameter has not been calculated because it is impossible to assume a perfect bicellar shape for this system.

Perspectives

The concept of magnetic orientation is not really new. However, a lack of methods to evaluate this phenomenon is evident in this area of research. Some authors (Forrest and Reeves, 1981) proposed order parameters to evaluate the extent of magnetic orientation, but these parameters are not really appropriate. More specifically, the measurement of the orientation via an order parameter such as S_2, as described above, is interesting but not complete. This order parameter could be indicative if a tilt of the bicelles or ellipsoids relative to the magnetic field axis is assumed. However, in general, it is more appropriate to suppose that

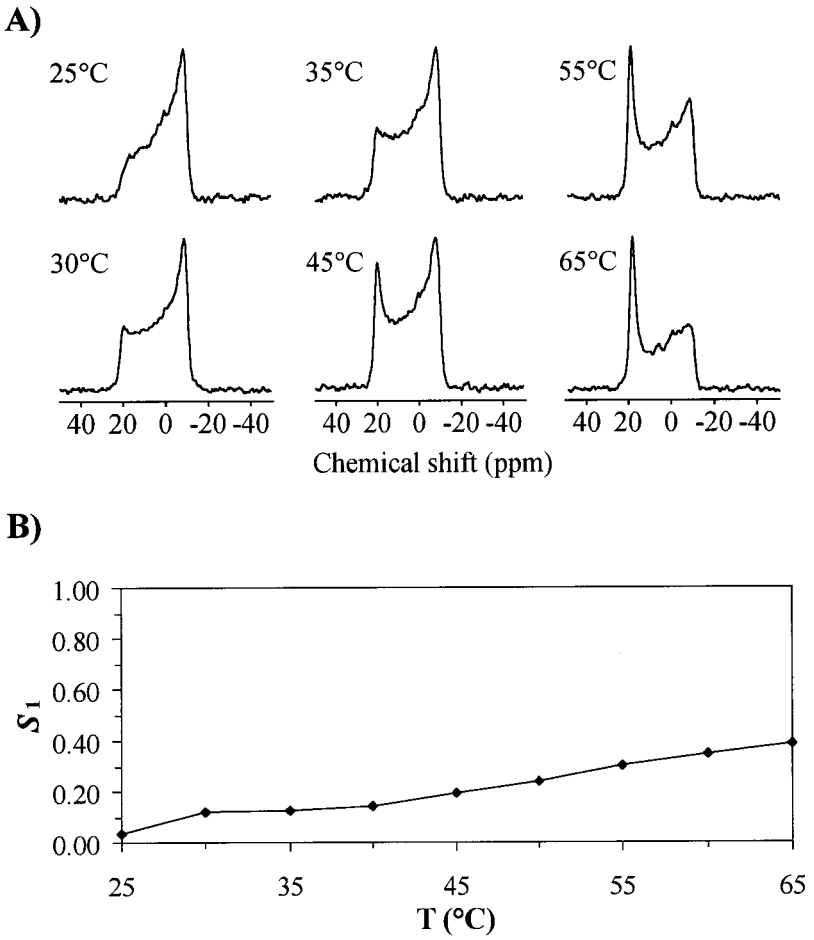

FIGURE 8 (*A*) Experimental ^{31}P NMR spectra and (*B*) S_1 as a function of temperature for the CL:ADM (2:1 molar ratio) system. A line broadening of 150 Hz was applied to these spectra.

the main axis of the deformed membrane is along or perpendicular to the magnetic field. In these cases, such order parameters will provide interesting information about the dynamics in the system. Therefore, the definition of another order parameter is really important. In the previous section, we proposed a new order parameter, S_1, derived from the measurement of the first spectral moment.

Spectral moments are well defined in the literature and are very easy to measure (Abragam, 1961). Therefore, an orientation parameter derived from this type of measurement is suitable. In fact, a change in the spectral lineshape due to partial orientation will affect the spectral moments. In principle, it could be possible to obtain orientation-dependent measurements from each of the spectral moments (M_1, M_2, M_3, M_4, . . .). However, the moments higher than the first one are very dependent on the spectral line broadening and only M_1 is not affected by symmetrical broadenings, such as Lorentzian or Gaussian broadenings. Therefore, only the use of M_1 is possible, even if M_1 is dependent on both the CSA and the isotropic chemical shift. With an evaluation of δ on a well-defined static spectrum and of δ_{iso} from a MAS spectrum, a really precise evaluation of S_1 is possible.

Because of the increasing use of both parallel and perpendicular bicelles as model membrane systems, the characterization of their shape and size is important. A model has recently been proposed based on ^{2}H NMR measurements and on the calculation of the relative areas of the planes and edges of the bicelles (Vold and Prosser, 1996). There are also extensive studies of the position of DHPC on the edges of the bicelles by ^{2}H and ^{31}P NMR spectroscopy (Sanders and Schwonek, 1992; Vold and Prosser, 1996). However, there are no simulated spectra of such bicelles in the literature, and therefore, an attempt to simulate both the perpendicular and parallel orientation of bicelles seems valuable.

The experimental spectra obtained for bicelles with their main axes oriented parallel to the magnetic field are in agreement with simulated spectra, suggesting the validity of this model. For perpendicular bicelles made of DMPC and DHPC, the presence of a second peak with a frequency different from δ_{iso} is a convincing proof of the validity of the bicelle model. In addition, both the relative intensity of the two peaks and the S_1 calculation give the same r shape parameter, which corresponds to the proposed diameter of the bicelles. Finally, the values of S_1 obtained for the phospholipids in the edges of the bicelles suggest that there is a phase separation between the lipids in the planes and the torus of the bicelles and that there is a fast exchange process between them. These results from ^{31}P NMR are in agreement with those obtained from ^{2}H NMR measurements (Vold and Prosser, 1996).

CONCLUSION

A new order parameter, S_1, has been proposed in the present study to obtain quantitative information about the orientation of phospholipidic systems. This new tool can be helpful both to investigate the shape of lipid membranes and to study the effect of several parameters (temperature, hydration, addition of proteins or drugs, etc.) on the orientation of phospholipidic systems. A second order parameter, S_2, can provide complementary information about the dynamics in an oriented system. ^{31}P NMR spectra have been simulated for both ellipsoid and bicellar systems in which the lipids are oriented either parallel or perpendicular to the external magnetic field. In addition, the S_1 and S_2 order parameters have been determined from the experimental spectra obtained for several systems.

More specifically, the general influence of temperature and concentration on orientation has been clearly demonstrated for pure DMPC multilamellar vesicles. In addition, the orientation and shape of bicelles made of DMPC:DHPC, in which the lipids are oriented perpendicular to the magnetic field, has been determined as a function of temperature. We have also shown in this study that the addition of GA to the DMPC:DHPC system induces an orientation of the lipids parallel to the magnetic field that seems to favor the formation of bicelles. Adriamycin also induces an orientation of the lipids at 0° relative to the magnetic field in CL bilayers, even in the absence of the short chain lipid DHPC. This indicates that a partial positive ordering can be obtained in lipid systems without the addition of paramagnetic ions and can be well characterized by the order parameter S_1 derived from the first spectral moment.

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